TAXONOMY OF INFRASPECIFC TAXA OF *ABIES LASIOCARPA*: LEAF ESSENTIAL OILS AND DNA OF *ABIES LASIOCARPA*, VAR. *BIFOLIA* AND VAR. *ARIZONICA*.

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ABSTRACT

Detailed analyses of the leaf essential oils of *Abies lasiocarpa* var. *lasiocarpa* (Olympic Peninsula, WA), *A. l.* var. *bifolia* (MT, UT) and *A. l.* var. *arizonica* (AZ) is presented to update the analyses of Hunt and von Rudloff (1979). The coastal alpine fir (*A. l.* var. *lasiocarpa*) is very strongly differentiated from all the Rocky Mtn. populations in having only a trace of camphene, no limonene, a large amount (53.3%) of β-phellandrene, no borneol, a large amount of piperitone (9.2%), methyl citronellate (0.4%), only a trace of bornyl acetate (0.1%), and a small amount of thymol (2.3%). The oil of *A. l.* var. *bifolia* has considerable amounts of (cpd., MT%, UT%): α-pinene (8.5, 3.4%), camphene (8.4, 15.0%), β-pinene (10.1, 16.5%), β-phellandrene (6.0, 4.1%), bornyl acetate (21.2, 24.9%) and thymol (3.5, 12.5%). The oil of *A. l.* var. *arizonica* has considerable amounts of α-pinene (9.2%), camphene (15.2%), β-pinene (24.0%), β-phellandrene (5.1%) and bornyl acetate (34.4%). The oil is differentiated by having no δ-3-carene, (E)-β-ocimene, trans-p-menth-2-en-1-ol, methyl citronellate, thymol, geranyl acetate or (E)-α-bisabolol. DNA sequencing of nrDNA, trnS-trnG, trnL-trnF, petN-psbM, and psbM-trnD yielded 5655 bp of data. NJ tree and SNPs analyses revealed the corkbark fir (*A. l.* var. *arizonica*) of the southern Rocky Mtns to be the most distinct of the taxa. Based on the oil and DNA data, there is support for the

recognition of *Abies lasiocarpa* var. *lasiocarpa* in the central Rocky Mountains and coastal North America and *Abies lasiocarpa* var. *arizonica* in the southern Rocky Mountains, but little support for the recognition of *A. l.* var. *bifolia. Phytologia 93(1):73-87 (April 1, 2011).*

KEY WORDS: *Abies lasiocarpa, var. bifolia, var. arizonica,* Alpine fir, *leaf* essential oils, DNA sequencing, SNPs, nrDNA, trnS-trnG, trnL-trnF, petN-psbM, psbM-trnD, taxonomy.

The taxonomy of the infraspecific taxa of *Abies lasiocarpa* (Hooker) Nuttall has been in a state of flux. Recently, Eckenwalder (2009) treated *A. lasiocarpa* as having three varieties: var. *lasiocarpa*, var. *bifolia* (A. Murray bis) Eckenwalder, and var. *arizonica* (Merriam) Lemmon. This is in deference to Hunt (1993) who recognized *A. lasiocarpa* in the coastal region and Cascades and northwesterly into coastal British Columbia and *A. bifolia*, inland in the Rocky Mtns. Hunt (1993) did not recognize var. *arizonica* (corkbark fir), but wrote that "the taxonomy of the corkbark fir ... is uncertain."

Zavarin et al. (1970) examined the monoterpenes from over 400 trees of *A. lasiocarpa* from throughout its range. They found the coastal populations to be high in β-phellandrene and low in limonene, with the inland, Rocky Mtn. populations high in limonene and low in β-phellandrene. The populations in the central and southern Rocky Mtns. appeared to have a different monoterpene pattern (Zavarin et al., 1970, Fig. 8). Of course, that pattern was based on the concentrations of only 3 key compounds: β-pinene, limonene and β-phellandrene.

Hunt and von Rudloff (1979) utilized the volatile leaf oils of *Abies* but published the composition of only the monoterpenes (Table 2, Hunt and von Rudloff, 1979). They found the leaf oils of the coastal populations to be high in β-phellandrene, low in limonene, with only trace amounts of camphene and bornyl acetate. In contrast, they found the Rocky Mtn. populations lower in β-phellandrene, higher in limonene, and with large amounts of camphene and bornyl acetate. Hunt and von Rudloff (1979) concluded that true *A. lasiocarpa* grew only in the northwest coastal mountains and the Rocky Mtn. alpine fir

was *A. bifolia*. This was reflected in Hunt's treatment of *Abies* in Flora of North America (Hunt, 1993).

The present study presents the first complete leaf essential oil (monoterpenes, sesquiterpenes, diterpenes) analyses of 'pure' *A. lasiocarpa* var. *lasiocarpa* (cf. popn. 54, Olympic Peninsula, WA, Hunt and von Rudloff, 1979), *A. l.* var. *bifolia* (cf. popn. 28, Glacier Park, MT, Hunt and von Rudloff, 1979), and *A. l.* var. *bifolia* from Utah as well as *A. l.* var. *arizonica,* Flagstaff, AZ.

Xiang et al. (2009) recently published a phylogeny of *Abies* based on nrDNA sequences. They found *A. lasiocarpa* in a clade with *A. balsamea* and *A. fraseri* sister to a clade of *A. koreana*, *A. veitchii* and *A. nephrolepis*. Other North American species such as *A. amabilis, A concolor, A. grandis, A. magnifica* and *A. procera* were in different clades (Xiang et al., 2009). However, it is unlikely that nrDNA data alone is sufficient to portray phylogenetic relationships. In this study, we present sequence data for nrDNA, petN-psbM, psbM-trnD, trnL-trnF and trnS-trnD.

MATERIALS AND METHODS

Leaf samples collected: *A. lasiocarpa* var. *lasiocarpa*: *Chris Earle-Adams 12315-12317*, Deer Park, Olympic National Park, Olympic Peninsula. 47.948826 N, 123.259027° W, 1643m, June 26, 2010, Clallam Co., WA;
A. lasiocarpa var. *bifolia*: *Adams 12400-12404*, Brighton Ski lodge parking lot. 40° 35' 48.76" N; 111° 35' 09.18" W, 2682m, Sept. 4, 2010, Salt Lake Co., UT; *Chris Earle, Adams 12413-12415*, 4 mi SW of St. Mary Lodge on US 89. Lat. 48.70142° N, 113.40362° W, 1730 m, Sept. 11, 2010, Glacier Co., MT.
A. lasiocarpa var. *arizonica, Thornburg-Adams 12388-12392*, 11 mi. NNW of Flagstaff, at Snowbowl Ski lodge parking lot, 35.32998° N; 111.71194' W, 2826m, Aug. 10, 2010, Coconino Co., AZ. All specimens are deposited in the BAYLU herbarium.

Isolation of Oils - Fresh leaves (200 g) were steam distilled for 2 h using a circulatory Clevenger-type apparatus (Adams, 1991). The oil samples were concentrated (ether trap removed) with nitrogen and the

samples stored at -20°C until analyzed. The extracted leaves were oven dried (100°C, 48 h) for determination of oil yields.

Chemical Analyses - Oils from 10-15 trees of each of the taxa were analyzed and average values reported. The oils were analyzed on a HP5971 MSD mass spectrometer, scan time 1 sec., directly coupled to a HP 5890 gas chromatograph, using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column (see Adams, 2007, for operating details). Identifications were made by library searches of our volatile oil library (Adams, 2007), using the HP Chemstation library search routines, coupled with retention time data of authentic reference compounds. Quantitation was by FID on an HP 5890 gas chromatograph using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column using the HP Chemstation software.

DNA Analysis - One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, then stored at -20° C until the DNA was extracted. DNA was extracted using the Qiagen DNeasy mini kit (Qiagen Inc., Valencia, CA).

The nrDNA region of *Abies lasiocarpa* proved to be too large (~1740 bp) to sequence by use of ITSA and ITSB (Fig. 1).

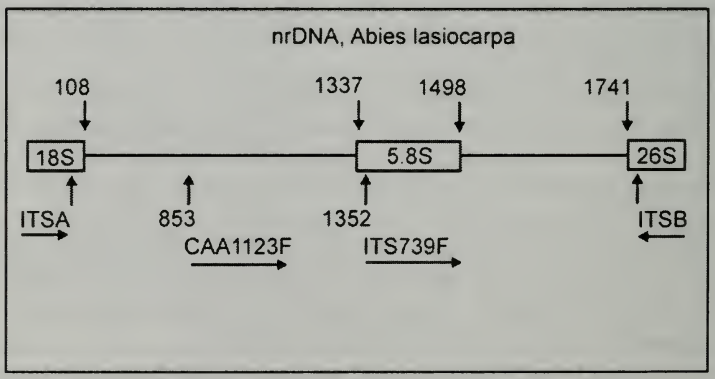

Figure 1. Diagram of the nrDNA region for *Abies lasiocarpa*.

Two addition primers were utilized:
CAA1123F: AC CTC CTA TGT CGG TTG TGC (Xiang et al. 2009)
ITS739F: AAC GGA TAT CTC GGC TCT, based on conserved sequences in the 5.8S region.

The trnC-trnD region of *Abies concolor* also proved to be large (~2400, Fig. 2). Due to the small area from trnC to petN, that region was

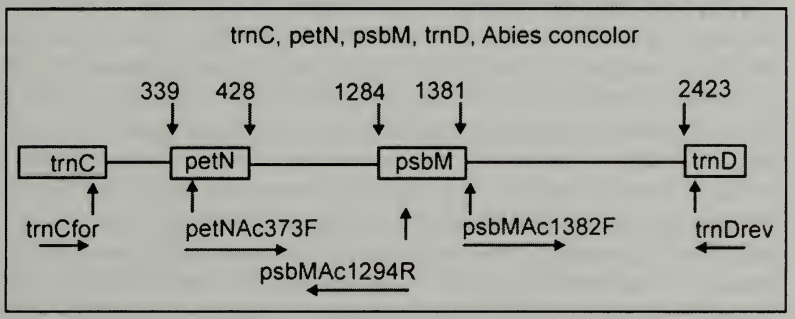

Figure 2. Diagram of the trnC-trnD region of *Abies concolor*.

skipped. Two regions were sequenced: petN-psbM and psbM-trnD using four primers (Fig. 2) based on sequences of *Abies* from GenBank:
petNAc373F: TGG TAG TTT TTA CAT TTT CC,
psbMAc1294R: TTA TCC CTT ACG TCA AAA CG
and
psbMAc1382F: AGA TCC ATG AAA TAG ATG TG
trnDrev: GGG ATT GTA GTT CAA TTG GT

Primers for trnL-trnF and trnS-trnG have been previously reported (Adams and Kauffmann, 2010).

PCR amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (cpDNA regions) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used) 1.8 µM each primer. The PCR reaction was subjected to purification by agarose gel electrophoresis (1.5% agarose, 70 v, 55 min.). In each case, the band was excised and purified using a Qiagen

QIAquick gel extraction kit. The gel purified DNA band with the appropriate primer was sent to McLab Inc. (South San Francisco) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.). Alignments and NJ trees were made using MAFFT (http://align.bmr.kyushu-u.ac.jp/mafft/).

Data Analysis - Terpenoids (as percent total oil) were coded and compared among the species by the Gower (1971) metric. Principal coordinate analysis was performed by factoring the associational matrix using the formulation of Gower (1966) and Veldman (1967). DNA data - Minimum spanning networks were constructed from SNPs data using PCODNA software (Adams et al., 2009). Associational measures were computed using absolute compound value differences (Manhattan metric), divided by the maximum observed value for that compound over all taxa (= Gower metric, Gower, 1971; Adams, 1975). Principal coordinate analysis was performed by factoring the associational matrix based on the formulation of Gower (1966) and Veldman (1967).

RESULTS AND DISCUSSION

In general, the leaf oils of *A. lasiocarpa* are dominated by monoterpenes with only small amounts of sesquiterpenes and diterpenes (Table 1). Table 1 shows the composition of the leaf essential oils of the four taxa. The monoterpenes are in good agreement with Zavarin et al. (1970) and Hunt and von Rudloff (1979). The coastal alpine fir (*A. l.* var. *lasiocarpa*) is very strongly differentiated from all the Rocky Mtn. populations in having only a trace of camphene, no limonene, a large amount (53.3%) of β-phellandrene, no borneol, a large amount of piperitone (9.2%), methyl citronellate (0.4%), only a trace of bornyl acetate (0.1%), a small amount of thymol (2.3%), the presence of α- and β-selinene, no (E)-β-bisabolene, and the presence of 10-epi-γ-eudesmol. Interestingly, the lasiocarpenes and juvabiones reported by Manville and Tracey (1989) in the wood of coastal *A. lasiocarpa* were not found in the leaf oils. Apparently, the wood and leaf oils differ in composition (similar to the situation in the Cupressaceae where the leaf and wood oils are quite different).

The oils of *A. l.* var. *bifolia* from Montana and Utah, are similar (Table 1), with considerable amounts of: α-pinene (8.5, 3.4%), camphene (8.4, 15.0%), β-pinene (10.1, 16.5%), β-phellandrene (6.0, 4.1%), bornyl acetate (21.2, 24.9%) and thymol (3.5, 12.5%). No unique compounds were found in these oils.

The oil of *A. l.* var. *arizonica* from Flagstaff, AZ (Table 1) has considerable amounts of α-pinene (9.2%), camphene (15.2%), β-pinene (24.0%), β-phellandrene (5.1%) and bornyl acetate (34.4%). The oil is differentiated by having no δ-3-carene, (E)-β-ocimene, trans-p-menth-2-en-1-ol, methyl citronellate, thymol, geranyl acetate or (E)-α-bisabolol. It contains only a trace of piperitone that is common in the other taxa (Table 1).

To visualize the overall similarities of the oils, principal coordinates ordination (PCO) was performed on a matrix of similarities based on 30 terpenoids (see Table 1). Three eigenroots were extracted and, of course, accounted for all the variation among the four populations. Ordination shows (Fig. 3) that each of the four populations are quite distinct with the oils of putative var. *bifolia* (MT and UT) being the most similar (0.644), followed by var. *arizonica* linking with UT (0.563), and lastly, the coastal, var. *lasiocarpa* population linking with UT (0.518).

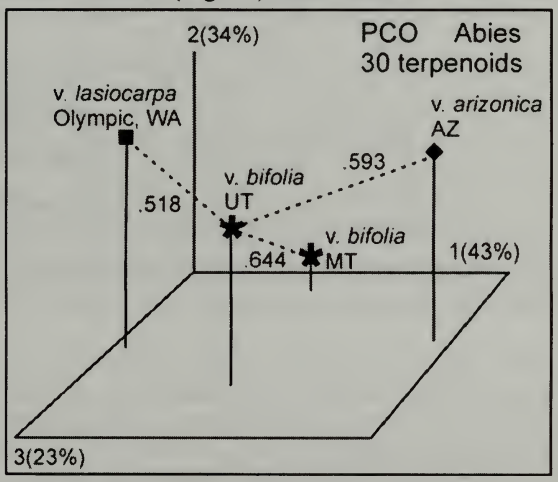

Figure 3. PCO based on 30 terpenoids. The dashed lines are the minimum spanning network (similarities).

Mapping the minimum spanning network (Fig. 4) enables one to obtain a geographic perspective. Clearly the differentiation between coastal alpine fir and Rocky Mtn. fir (cf. Hunt and von Rudloff, 1979) is well supported. It is interesting to note that *A. lasiocarpa*, Olympic Peninsula, is a bit more similar to var. *bifolia* from Utah (0.518) than to Montana (0.484). The linkage of var. *arizonica* with Utah reconfirms the trend found by Zavarin et al. (1970).

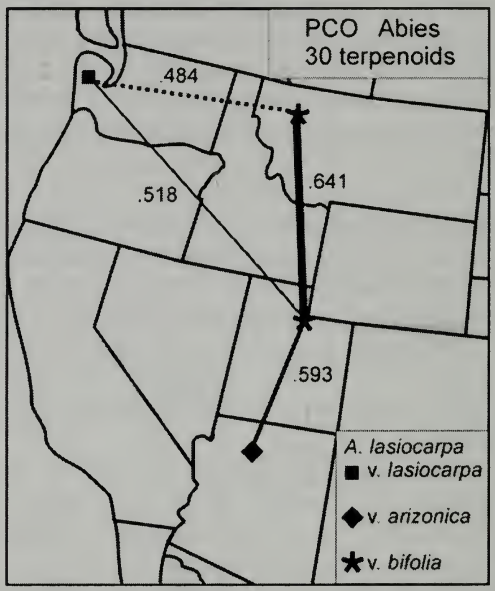

Figure 4. Minimum spanning network based on 30 terpenoids. The dotted line shows the second nearest link for WA (to MT).

Analyses of the concatenated set of nrDNA, trnS-trnG, trnL-trnF, petN-psbM and psbM-trnD sequences resulted in 5655 bp of data. A NJ analysis (Fig. 5) shows each of the four populations of *A. lasiocarpa* in well supported clades. The clade of *A. lasiocarpa* var. *arizonica* is quite distinct (Fig. 5).

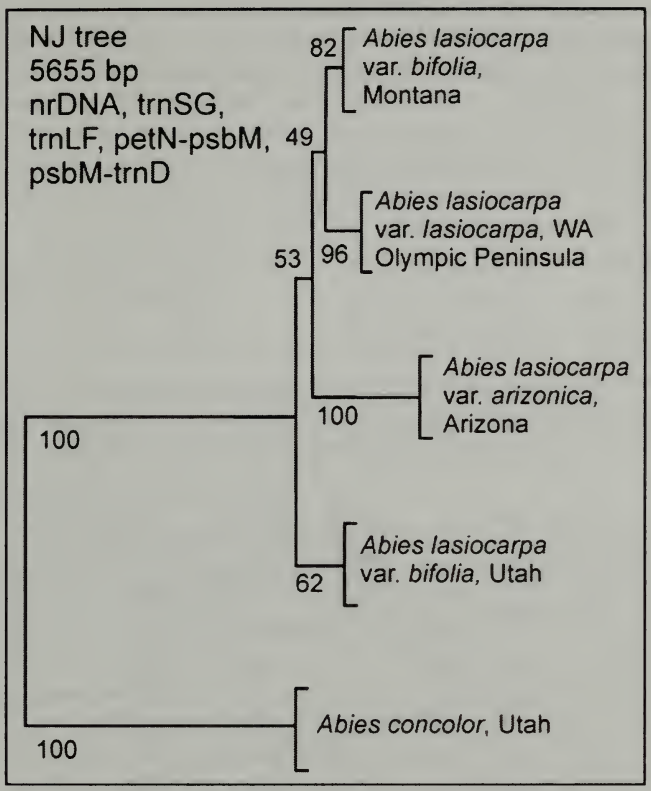

Figure 5. NJ tree based of nrDNA (nuclear) plus cpDNA (trnS-trnG, trnL-trnF, petN-psbM, psbM-trnD). Numbers at the branch points are bootstrap values as percents.

Table 2 shows a summary of the variation in nrDNA, trnS-trnG, trnL-trnF, petN-psbM, and psbM-trnD sequences. Of the large number (135) of substitutions found in nrDNA, 125 were found only in *A. concolor* and this appears to be due to poor alignment as the nrDNA sequence in *A. concolor* contained 11 indels and appeared to be rearranged, making it very difficult to align with *A. lasiocarpa.*

Table 2. Summary of variation found in nrDNA, trnS-trnG, trnL-trnF, petN-psbM, and psbM-trnD sequences. S = population useful base substitution, i = indel, u = unique substitution, found in only one sample.

DNA	*A. concolor* + *lasiocarpa*	*A. lasiocarpa* only
nrDNA	2047bp, 135S, 11i, 0u	1821bp, 10S, 0i, 1u
trnS-trnG	892bp, 10S, 2i, 2u	892bp, 0S, 0i, 2u
trnL-trnF	958bp, 6S, 2i, 0u	958bp, 0S, 1i, 0u
petN-psbM	870bp, 13S, 2i, 0u	868bp, 1S, 0i, 2u
psbM-trnD	927bp, 16S, 5i, 0u	887bp, 2S, 3i, 0u

Construction of a minimum spanning network (in which indels are utilized) shows the overall relationships on a geographic map (Fig. 6). The *A. lasiocarpa* var. *bifolia* Montana population differs by only 3 SNPs (out of 5633bp) from the Utah population and 3 SNPs from the *A. l.* var. *lasiocarpa* Olympic population. This is certainly a rather minor amount of differentiation. The *A. l.* var. *arizonica* population differs by 8 SNPs from var. *bifolia*, Montana and 9 SNPs from var. *bifolia*, Utah and 11 SNPs from var. *lasiocarpa*, Olympics (Fig. 6).

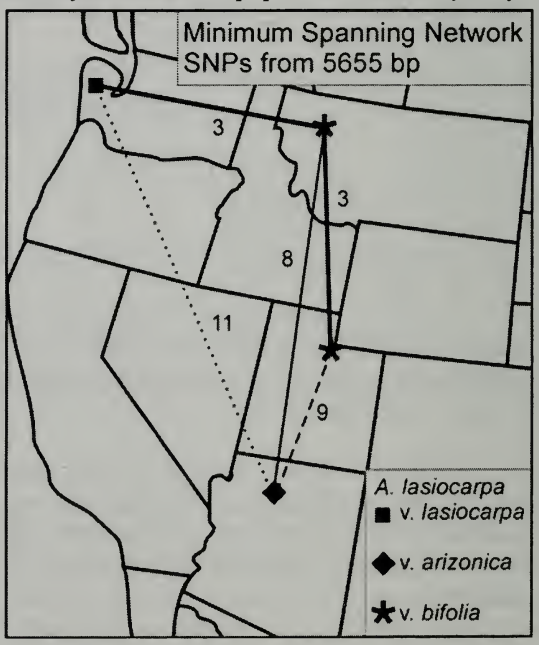

Figure 6. Minimum spanning network based on 5655 bp of sequence data. The numbers next to the links are the number of SNPs (including indel information).

CONCLUSIONS

The leaf essential oil compositions from these four populations are remarkably differentiated and support the recognition of *A. lasiocarpa* var. *lasiocarpa* from the coastal region of the northwestern US and Canada, and *Abies l.* var. *bifolia* from the northern Rocky Mtns. as suggested by Hunt and von Rudloff (1979) and Manville and Tracey (1989). In addition, the corkbark fir (*A. l.* var. *arizonica*) of the southern Rocky Mtns. is very distinct in its leaf oil and its recognition is supported by these data.

The DNA sequence data is congruent with the oil data of corkbark fir (*A. lasiocarpa* var. *arizonica*) showing it is quite distinct. However, the DNA data gives less support (than the oil data) for the recognition of *A. l.* var. *bifolia* as being distinct from *A. l.* var. *lasiocarpa.*

The conflict between essential oils data and DNA sequence information is not new in conifers. Adams et al. (2005) found prefect taxonomic concordance between nrDNA, RAPDs, leaf essential oils and morphology separating *Juniperus deltoides* R. P. Adams and *J. oxycedrus*. In contrast, Adams et al. (2008) found considerable differences in the classifications of *Juniperus excelsa* M.-Bieb. and *J. polycarpos* K. Koch. varieties between nrDNA, trnC-trnD SNPs and the leaf essential oil data sets. In general, Adams (2008) notes that the leaf essential oils are most useful below the specific level in the study of geographic variation. It seems likely that the alpine fir in the Pacific northwest region (*A. lasiocarpa* var. *lasiocarpa* in this study) is subjected to a quite different set of selection pressures in regards to diseases, insects and herbivores than faced by alpine fir in the Rocky Mountains. Thus, the leaf oils are likely revealing future speciation processes that are not yet apparent in the 'neutral' mutations in the gene introns utilized in this study.

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Table 1. Comparison of leaf oil compositions of *Abies lasiocarpa*. Olym = *A. lasiocarpa* v. *lasiocarpa*, Olympic Peninsula, WA, Mont = *A. l.* v. *bifolia*, Montana, Utah = *A. l.* v. *bifolia*, Utah, Ariz = *Abies l.* v. *arizonica*, Arizona. Compounds in bold face appear to separate the taxa. Compositional values less than 0.1% are denoted as traces (t). Unidentified components less than 0.5% are not reported. RI is the Kovat's Index using a linear approximation on DB-5 column. *= cpds used for PCO (30 cpds.)

RI	compound	Olym	Mont	Utah	Ariz
884	santene*	t	1.2	1.0	0.4
921	tricyclene*	t	0.7	1.1	0.9
924	α-thujene	t	-	-	-
932	**α-pinene***	**4.2**	**8.5**	**3.4**	**9.2**
946	**camphene***	**0.1**	**8.4**	**15.0**	**15.2**
969	sabinene	t	0.6	t	0.4
974	β-pinene*	16.6	10.1	16.5	24.0
988	myrcene*	1.6	0.9	0.6	0.7
1002	α-phellandrene*	0.6	0.3	0.3	0.1
1008	**δ-3-carene***	**0.7**	**4.1**	**0.3**	**-**
1014	α-terpinene	0.2	0.2	0.2	t
1020	p-cymene	t	t	t	t
1024	**limonene***	**-**	**17.6**	**8.4**	**2.5**
1025	**β-phellandrene***	**53.3**	**6.0**	**4.1**	**5.1**
1038	2-heptyl acetate	-	t	0.1	-
1044	**(E)-β-ocimene***	**0.5**	**0.3**	**0.1**	**-**
1054	γ-terpinene	0.2	0.2	0.1	0.1
1065	cis-sabinene hydrate	t	t	t	t
1086	terpinolene*	1.0	1.3	0.8	0.4
1095	6-camphenone	-	-	-	t
1095	linalool	0.2	0.4	0.3	0.2

RI	compound	Olym	Mont	Utah	Ariz
1098	trans-sabinene hydrate	-	t	-	-
1118	endo-fenchol	-	-	-	t
1118	**cis-p-menth-2-en-1-ol***	**0.9**	**0.5**	**1.0**	**t**
1122	α-campholenal	t	-	t	t
1135	trans-pinocarveol	-	-	-	t
1136	**trans-p-menth-2-en-1-ol***	**0.8**	**0.5**	**0.8**	**-**
1141	camphor*	0.1	0.3	0.4	0.7
1145	camphene hydrate	-	0.2	0.2	0.1
1148	citronellal	-	t	0.1	t
1160	pinocarvone	t	-	-	-
1165	p-mentha-1,5-dien-8-ol	-	0.1	t	-
1165	**borneol***	**-**	**0.4**	**0.4**	**2.9**
1172	cis-pinocamphone	-	-	-	0.1
1174	terpinen-4-ol*	0.4	0.6	0.3	0.3
1186	α-terpineol*	0.3	0.5	0.5	0.3
1195	cis-piperitol	0.2	0.1	0.2	-
1195	myrtenal	-	-	-	t
1195	myrtenol	0.2	0.1	0.3	t
1204	verbenone	-	-	-	t
1207	trans-piperitol*	0.4	0.3	0.4	-
1218	endo-fenchyl acetate	-	t	0.1	-
1223	citronellol*	t	0.4	t	0.4
1232	thymol, methyl ether*	1.0	0.6	t	t
1249	**piperitone***	**9.2**	**4.9**	**1.4**	**t**
1257	**methyl citronellate***	**0.4**	**t**	**t**	**-**
1274	neo-isopulegol acetate	-	-	t	t
1287	**bornyl acetate***	**0.1**	**21.2**	**24.9**	**34.4**
1289	**thymol***	**2.3**	**3.5**	**12.5**	**-**
1298	trans-pinocarvyl acetate	-	-	-	t
132	cis-piperityl acetate	t	-	-	-
1350	citronellyl acetate	t	t	0.1	-
1359	neryl acetate	t	-	-	-
1379	**geranyl acetate***	**1.1**	**0.1**	**0.4**	**-**
1387	β-bourbonene	-	t	-	-
1389	longifolene	-	t	0.2	-
1400	β-longipinene	-	-	-	t

RI	compound	Olym	Mont	Utah	Ariz
1436	isobazzanene	-	0.1	-	-
1458	allo-aromadendrene	t	-	-	-
1480	allo-aromadendr-9-ene	-	0.1	t	-
1489	**β-selinene**	**0.1**	**t**	**t**	**t**
1493	δ-decalactone	-	0.2	t	t
1498	**α-selinene**	**0.1**	**-**	**-**	**-**
1500	α-muurolene	-	t	-	-
1505	β-bisabolene	0.1	t	t	-
1539	**(E)-β-bisabolene***	**-**	**0.7**	**0.2**	**0.1**
1561	(E)-nerolidol	0.1	-	0.1	t
1622	**10-epi-γ-eudesmol**	**0.2**	**-**	**-**	**-**
1656	valerianol	0.3	t	t	-
1658	neo-isointermedeol	-	t	t	-
1683	**(E)-α-bisabolol***	**0.5**	**0.3**	**0.5**	**-**
1981	isopimara-8(14),15-diene	t	t	t	-
1987	manoyl oxide	t	0.1	t	t
2014	palustradiene(=abieta-8, 13-diene) *	0.2	0.7	0.5	0.1
2055	abietatriene*	0.2	0.4	0.7	0.1
2087	abietadiene	t	0.1	0.1	t
2149	abienol	0.2	t	t	t
2300	tricosane(C23)	-	t	t	0.1
2313	abietal	-	0.1	t	t